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Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding

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Prions are the infectious agents responsible for transmissible spongiform encephalopathies. The principal component of prions is the glycoprotein PrP^{Sc}, which is a conformationally modified isoform of a normal cell-surface protein called PrP^C (ref. 1). During the time between infection and the appearance of the clinical symptoms, minute amounts of PrP^{Sc} replicate by conversion of host PrP^C, generating large amounts of PrP^{Sc} aggregates in the brains of diseased individuals. We aimed to reproduce this event *in vitro*. Here we report a procedure involving cyclic amplification of protein misfolding that allows a rapid conversion of large excess PrP^C into a protease-resistant, PrP^{Sc}-like form in the presence of minute quantities of PrP^{Sc} template. In this procedure, conceptually analogous to polymerase chain reaction cycling, aggregates formed when PrP^{Sc} is incubated with PrP^C are disrupted by sonication to generate multiple smaller units for the continued formation of new PrP^{Sc}. After cyclic amplification more than 97% of the protease-resistant PrP present in the sample corresponds to newly converted protein. The method could be applied to diagnose the presence of currently undetectable prion infectious agent in tissues and biological fluids, and may provide a unique opportunity to determine whether PrP^{Sc} replication results in the generation of infectivity *in vitro*.

To evaluate the cyclic amplification procedure (Fig. 1), we diluted brain homogenate from scrapie-affected hamsters until the signal of PrP^{Sc} was barely detectable by immunoblot after digestion with proteinase K (Fig. 2a, lane 1). The same dilution of scrapie brain homogenate was incubated with brain homogenate from healthy hamsters as a source of PrP^C. After incubation, an increase in the signal of a protease-resistant PrP^{Sc}-like protein (PrPres) with relative molecular mass 27,000–30,000 (*M_r* 27–30K) could be detected (Fig. 2a, lane 2). Using the same conditions, but subjecting the mixture to five cycles of incubation–sonication, the amount of PrPres dramatically increased (Fig. 2a, lane 3). This process of *in vitro* replication of protein conformation is called protein-misfolding cyclic amplification (PMCA). To estimate the rate of amplification, we performed densitometric analysis of immunoblots from five independent experiments done under the same conditions. The average amplification rate was 57.9 ± 19.9 , indicating that after five PMCA cycles the newly converted protein corresponds to 97.4–98.7% of the total amount of PrPres. This proportion can be further increased by subjecting the sample to a larger number of amplification cycles (see below). To rule out artefacts of protein blotting, rat brain homogenate was added to the control sample of scrapie hamster brain homogenates to achieve equal protein concentrations (Fig. 2b). Only the hamster-derived protein was measured as the

antibody used to reveal PrPres in the immunoblot does not react with rat prion protein. The conversion was dependent on the presence of PrP^{Sc}, as no PrPres formation was observed when the normal hamster brain homogenate was incubated alone, under the same conditions, with or without sonication (Fig. 2c, lanes 2 and 3). In addition, the increase in PrPres signal was not due to higher reactivity of initial PrP^{Sc} as a result of the treatment, because incubation–sonication of the material in the absence of a source of PrP^C resulted in no change in the signal (Fig. 2c, lanes 5 and 7). We noted that in the control experiment in which brain homogenates containing PrP^C and PrP^{Sc} were incubated, but not subjected to PMCA (Fig. 2a, lane 2), PrPres formation was greater than that reported previously using purified proteins^{2,3} or cell lysates⁴. The higher efficiency of conversion might be due to the presence of additional factors present in the brain homogenate that catalyse the conversion^{4,5}. The amplification system could be useful to isolate such additional factors by providing an assay to monitor their activity.

To evaluate the minimum PrP^{Sc} concentration needed to trigger amplification and enhanced detection of abnormal protein, we serially diluted brain homogenate from hamsters with scrapie directly into brain homogenate from healthy hamsters and assayed for PrPres signal with or without incubation–sonication cycles. Without PMCA, the PrPres signal diminished progressively until it

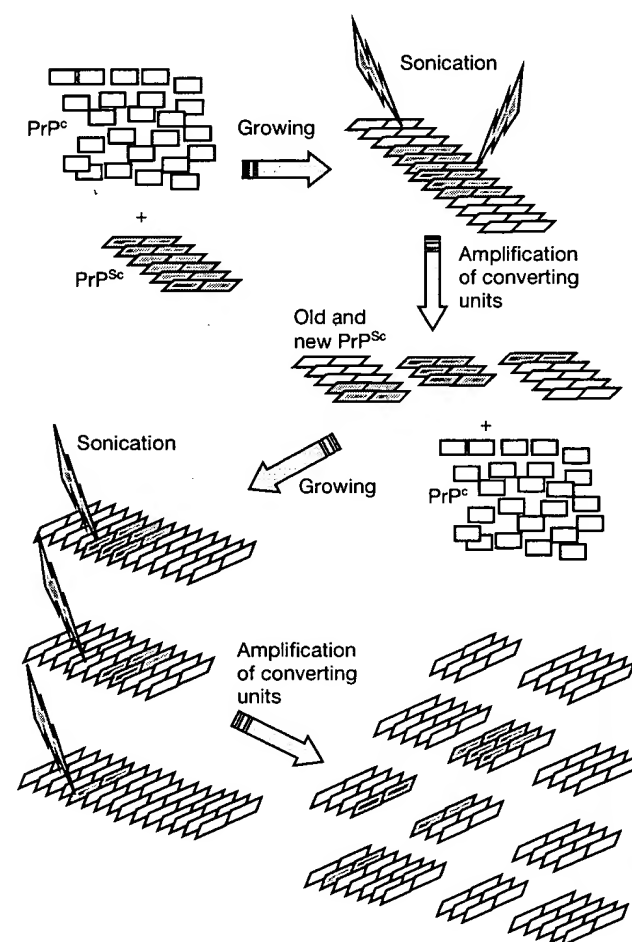


Figure 1 Diagrammatic representation of the PMCA procedure. Amplification is based on multiple cycles of PrP^{Sc} incubation in the presence of excess PrP^C followed by sonication. During the incubation periods, the size of oligomeric PrP^{Sc} is increased by incorporation of PrP^C into the growing aggregate, while during sonication the aggregates are disrupted, producing an expanded population of converting units.

was not longer detectable at ~640-fold dilution (Fig. 3a, c). By contrast, when parallel samples were subjected to 10 PMCA cycles, PrPres was readily detected at this dilution (Fig. 3b, c). Indeed, PrPres was detected even after >10,000-fold dilution under these conditions. On the basis of our estimation of PrP^{Sc} detection by immunoblot of known amounts of recombinant hamster PrP, the minimum amount of PrP^{Sc} detectable under these conditions is ~6–12 pg or $0.2\text{--}0.4 \times 10^{-15}$ mol. The amount of newly converted PrPres is ~250 pg or 8.3×10^{-15} mol. This detection limit was calculated using low-sensitivity immunoblot assays and therefore it might be further decreased, to reach a similar or even better sensitivity than the biological assay of infectivity, by using detection systems with higher sensitivity and/or by performing a larger number of amplification cycles (see below).

To determine the effect of different numbers of PMCA cycles on PrPres formation, we diluted scrapie brain homogenate 1,000-fold and incubated it with an excess of healthy hamster brain homogenate. Samples were treated with 0, 5, 10, 20 or 40 cycles and the resultant PrPres signal determined by immunoblot. As anticipated, the levels of PrPres increased with the number of cycles (Fig. 4a). Control samples containing identical mixtures were incubated for the same time, but without sonication. No increase in PrPres signal strength was detected for these samples (Fig. 4b). The observed increase in PrPres per cycle fitted an exponential curve ($r^2 = 0.973$, Fig. 4c), limited by an underestimation of the amplified material at higher cycle numbers. Samples subjected to more than 20 cycles

formed strong aggregates that could not be converted to monomeric protein during electrophoresis and hence were excluded from the quantification.

Propagation of transmissible spongiform encephalopathies (TSE) is believed to depend on the replication of PrP^{Sc} at the expense of the normal protein^{1,6}. Although the molecular details of the replication process are not completely known, it involves changes in conformation that are stabilized upon protein oligomerization^{6–8}. It is assumed that this process occurs *in vivo*, taking months or even years after infection of the host for PrP^{Sc} replication to progress sufficiently to trigger appearance of the disease. PrP replication has been performed *in vitro* in a cell-free system, by mixing purified PrP^C with an equimolar concentration of partially denatured PrP^{Sc} (ref. 2). This system has been used to study the molecular mechanism of PrP conversion⁹, the sequence specificity of PrP^{Sc} formation⁹, and to identify and evaluate inhibitors of PrP transformation^{10,11}. However, the efficiency of the cell-free conversion is low, as the amount of newly converted protein is much less than the initial concentration of PrP^{Sc} needed to trigger the conversion reaction. This problem has precluded the study of the structural and infectious properties of the newly converted PrPres⁶. Our aim was to develop a system that mimics the replication of prions in the body during infection, by starting with undetectable concentrations of PrP^{Sc}, mixing it with a large excess

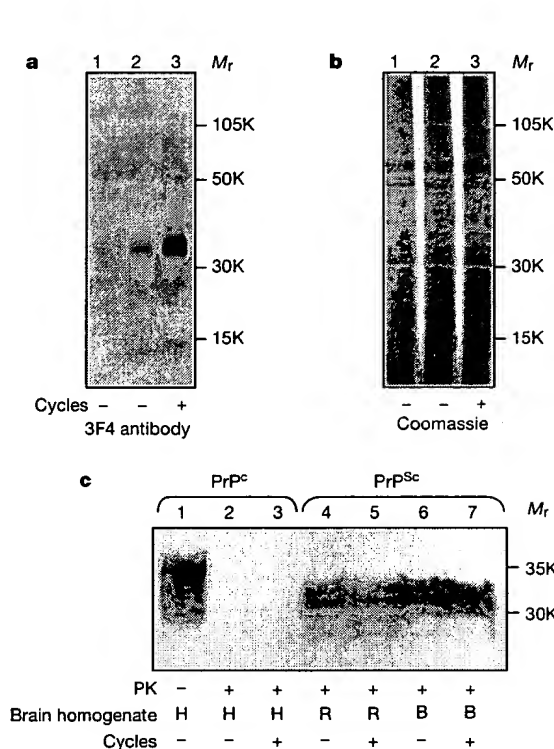


Figure 2 Amplification of PrP^{Sc} by sonication cycles. Scrapie hamster brain homogenate was diluted 500-fold into 10% healthy rat brain homogenate (lane 1 in a and b, control experiment) or into 10% healthy hamster brain homogenate (lanes 2 and 3 in a and b). Samples were incubated during 5 h without (lanes 1 and 2 in a and b) or with (lane 3 in a and b) five PMCA cycles. All samples were treated with proteinase K (PK). Samples were immunoblotted with the 3F4 anti-PrP antibody (a) or loaded onto a gel and stained for total protein with Coomassie blue (b). c, Control experiments with brain homogenate of healthy hamsters containing PrP^C (lanes 1–3) or brain homogenate of scrapie-affected hamsters containing PrP^{Sc} (lanes 4–7), were diluted 100-fold in healthy hamster brain homogenate (H), healthy rat brain homogenate (R) or PBS buffer (B), as indicated. Samples in lanes 1, 2, 4 and 6 were incubated for 5 h at 37 °C; samples in lanes 3, 5 and 7 were subjected to five PMCA cycles.

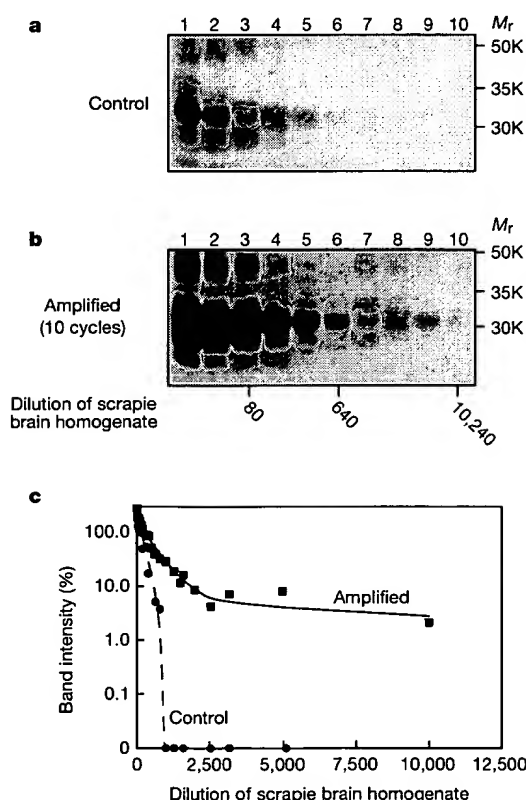


Figure 3 Sensitivity of the PMCA system. a, Scrapie hamster brain homogenate diluted serially into 5% rat brain homogenate (control). b, Two-fold serial dilutions of the scrapie brain homogenate into a 5% healthy hamster brain homogenate and subjected to 10 PMCA cycles. All samples in a and b were treated with proteinase K. The dilutions of scrapie brain homogenate in lanes 1–10 of a and b were 20, 40, 80, 160, 320, 640, 1,280, 2,560, 5,120 and 10,240, respectively. c, Densitometric quantification of five independent experiments to evaluate the sensitivity threshold of the cyclic amplification under these conditions. To normalize differences between experiments, the band intensity at each dilution is expressed as a percentage of the PrPres band intensity obtained at a 100-fold dilution of the scrapie brain homogenate without any treatment.

of PrP^C, and finishing the reaction with most of the molecules in the altered conformation. Using the PMCA procedure we estimate that the initial amount of PrP^{Sc} corresponds to less than 3% of the total concentration of PrPres produced at the end of the conversion. The amplification procedure requires the presence of several factors: exogenous PrP^{Sc} acting as a template for the conversion; PrP^C that serves as a substrate; and unknown factors present in brain homogenate that catalyse the reaction (G.P.S. and C.S., unpublished observations). The strategy to speed up the conversion, and thus reproduce in a few hours *in vitro* a process that takes months or years for completion *in vivo*, was to perform cycles of incubation-sonication. It has been proposed that the infective unit of PrP^{Sc} is a β -sheet-rich oligomer that converts the normal protein by integrating it into the growing aggregate, where it acquires the properties associated with the abnormal protein^{12,13}. After incubation of the two forms of PrP, the oligomeric species increase in size by recruiting and transforming PrP^C molecules. In the PMCA system, PrP^{Sc} is incubated with an excess of non-pathogenic conformer to enlarge the oligomeric converting units, followed by a sonication step to break down the aggregates into smaller units, each of which is capable of initiating further rounds of growth (Fig. 1). Our results demonstrate for the first time that the folding and biochemical properties of a protein can be transferred cyclically to other protein molecules, resulting in amplification of protein conformation in a manner conceptually analogous to DNA amplification by polymerase chain reaction (PCR).

A highly debated issue in the field of TSE is the nature of the infectious agent¹⁴⁻¹⁶. Strong evidence supports the 'protein-only' hypothesis of TSE propagation^{1,6}. However, the conclusive proof of

de novo production of prions is still lacking^{6,15}. Our results show for the first time a high-efficiency replication of PrP conformation *in vitro*, mimicking the process of PrP^{Sc} replication thought to occur *in vivo* during the disease. Therefore, our findings reinforce one of the aspects of the prion hypothesis, that minute amounts of PrP^{Sc} have the ability to replicate its biochemical properties by converting large amounts of PrP^C. Cyclic amplification of PrP^{Sc} in the presence of PrP^C provides a unique opportunity to generate infectivity *in vitro*. The protein generated with the PMCA procedure shares several of the biochemical properties typical of PrP^{Sc} extracted from the brains of animals with scrapie, including protease-resistance, detergent insolubility (data not shown) and the ability to further convert PrP^C *in vitro*. Because of the high yield of conversion, the infectious properties of the newly generated PrP^{Sc}-like protein can be tested and distinguished from the small amount of PrP^{Sc} used to begin the conversion reaction. We are currently determining the infectious properties of the PrP^{Sc}-like protein generated by PMCA.

The definitive diagnosis of TSE is based on the demonstration of PrP^{Sc} in brain tissue of the affected host¹⁷. Indeed, PrP^{Sc} is the only validated surrogate marker for the disease and the only known component of the TSE infectious agent¹. The lack of a prion-specific nucleic acid prevents the use of highly sensitive PCR-based diagnostic tests. Given the recent widespread distribution of cases of bovine spongiform encephalopathy (BSE) in Europe and the strong arguments linking it to variant Creutzfeldt-Jakob disease (CJD) in humans^{18,19}, development of a sensitive diagnostic test that can reliably identify the disease in animals and people during the pre-symptomatic period is a top priority²⁰. Currently available methods to detect PrP^{Sc} are limited by the amount of the abnormal protein

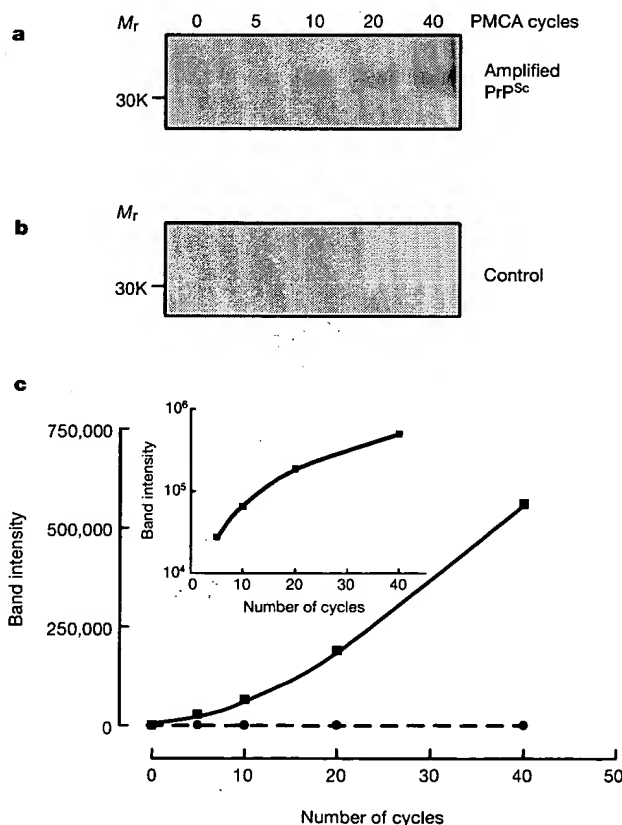


Figure 4 Relationship between the extent of the conversion and the number of amplification cycles. A 1,000-fold dilution of hamster scrapie brain homogenate was made into 5% healthy hamster brain homogenate. Aliquots were subjected to 0, 5, 10, 20 or 40 PMCA cycles (a) or incubated for the same times, but without sonication (b). All

samples were treated with proteinase K and PrPres signal was evaluated by immunoblot. To appreciate more clearly the differences in the band intensity, one tenth of the samples were loaded in the gel. c, Densitometric quantification of the blots in a (PMCA, solid line) and b (control, dotted line); the inset shows the same data in a logarithmic scale.

present in the tissues²¹. This limitation results in late-stage diagnosis, in most cases from brain tissue obtained at autopsy^{20,21}. However, as shown by infectivity studies, the infectious agent is present pre-symptomatically in many tissues in addition to the brain^{21,22}. Thus, early detection of PrP^{Sc} from non-brain sources should be possible with highly sensitive methods²¹. Our findings constitute a strategy to detect low quantities of PrP^{Sc}, by means of amplifying undetectable amounts of the protein to a detectable level. The PMCA procedure can be combined with any of the existing detection systems to reach a further reduction of detection threshold. Preliminary results indicate that the PMCA system can also be applied to human brain samples obtained from sporadic CJD (G.P.S. and C.S., unpublished observations). Therefore, the PMCA method opens a new possibility for TSE diagnosis that could be applied to systemic tissues or fluids during the pre-symptomatic phase of the disease. □

Methods

Preparation of brain homogenates

Brains from healthy rats (F344) and Syrian golden hamsters healthy or infected with the adapted scrapie strain 263 K were obtained after decapitation and immediately frozen in dry ice and kept at -80 °C until used. Brains were homogenized in PBS buffer containing protease inhibitors (Complete cocktail from Boehringer Mannheim) at a 1 × final concentration. Detergents (0.5% Triton X-100, 0.05% SDS, final concentrations) were added and samples clarified with low-speed centrifugation (1,000g) for 1 min, using an Eppendorf 5415 centrifuge.

Cyclic amplification

Serial dilutions of the scrapie brain homogenate were made directly in the healthy brain homogenate. We incubated 60 µl of these dilutions at 37 °C with agitation. Every hour one cycle of sonication (five pulses of 1 s each) was done using a microsonicator (Bandelin Electronic, Sonopuls) with the probe immersed in the sample and the power setting fixed at 40%. These cycles were repeated 5–40 times.

Detection of PrP^{Res}

The samples were digested with proteinase K (100 µg ml⁻¹ for 60 min at 37 °C) and the reaction was stopped with 50 mM phenyl-methyl sulphonyl fluoride. Samples were separated by SDS-PAGE and electroblotted into nitrocellulose membrane in 3-(cyclohexylamino)-1-propane sulphonic acid or Tris-glycine transfer buffer with 10% methanol during 45 min at 400 mA. For immunoblotting, the membranes were blocked with 5% non-fat milk and incubated for 2 h with the monoclonal antibody 3F4 (ref. 23) (1:50,000). Four washes of 5 min each were performed with PBS and 0.3% Tween20 before incubation with secondary anti-mouse antibody labelled with horseradish peroxidase (1:5,000) for 1 h. After washing, the reactivity in the membrane was developed with an ECL Chemiluminescence Kit (Amersham) according to the manufacturer's instructions. Densitometric analyses of western blots were performed with the program SigmaGel v1.0 (Jandel Scientific). The concentration of PrP^{Sc} was estimated by densitometric analysis and comparison with pure recombinant hamster PrP, the concentration of which was determined by amino-acid analysis.

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Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase

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Bacterial cells sense their population density through a sophisticated cell–cell communication system and trigger expression of particular genes when the density reaches a threshold. This type of gene regulation, which controls diverse biological functions including virulence, is known as quorum sensing^{1,2}. Quorum-sensing signals, such as acyl-homoserine lactones (AHLs), are the essential components of the communication system. AHLs regulate virulence gene expression in a range of plant and animal (including human) bacterial pathogens^{3–9}. AHL-producing tobacco restored the pathogenicity of an AHL-negative mutant of *Erwinia carotovora*¹⁰. Different bacterial species may produce different AHLs, which vary in the length and substitution of the acyl chain but contain the same homoserine lactone moiety. Here we show that the acyl-homoserine lactonase (AHL-lactonase), a new enzyme from *Bacillus* sp.¹¹, inactivates AHL activity by hydrolysing the lactone bond of AHLs. Plants expressing AHL-lactonase quenched pathogen quorum-sensing signalling and showed significantly enhanced resistance to *E. carotovora* infection. Our results highlight a promising potential to use quorum-sensing signals as molecular targets for disease control, thereby broadening current approaches for prevention of bacterial infections.

Target genes regulated by AHLs are extremely varied and regulatory mechanisms are probably diversified^{12–14}; however, the general mechanism of AHL-mediated quorum-sensing signalling is highly conserved. In general, each bacterial cell produces a basal level of AHLs that move in and out of cell membranes through diffusion or active transportation (ref. 15 and L.-H.Z., unpublished data). When AHLs reach a threshold concentration owing to